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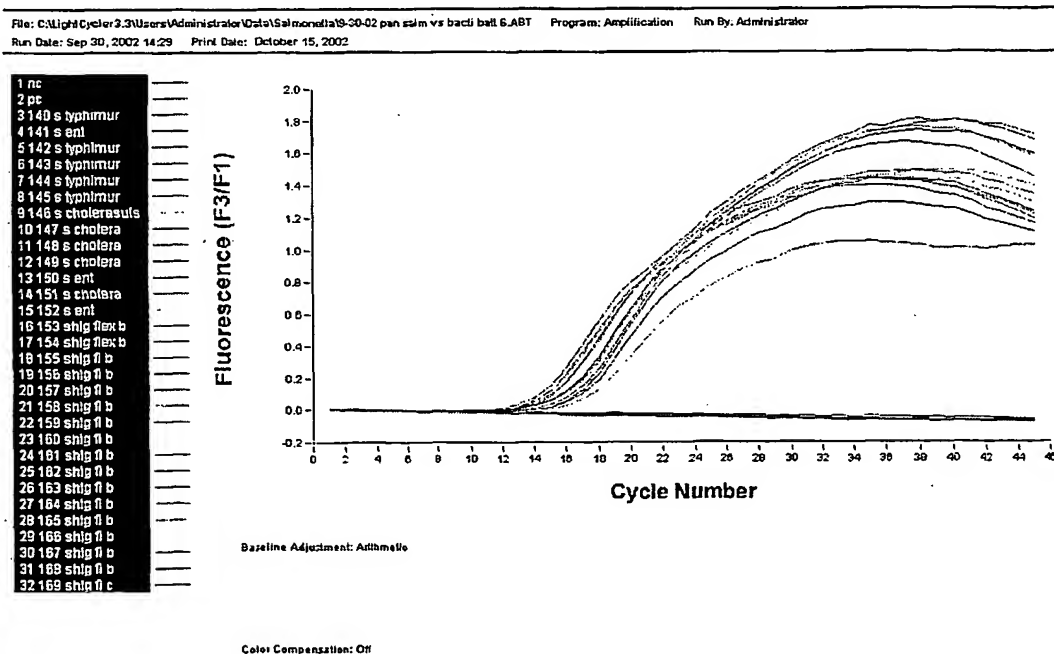
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[Continued on next page]

**(54) Title:** *SALMONELLA* DETECTION IDENTIFICATION



(57) **Abstract:** A nucleic acid specific for use in detecting and differentiating *Salmonella* from other bacteria comprises at least 10 contiguous nucleotides and is capable of selectively hybridizing to at least a portion of the *prg* gene of *Salmonella*.

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## SALMONELLA DETECTION IDENTIFICATION

### RELATED APPLICATION

This application claims priority from U.S. provisional patent application Serial No. 60/466,398, filed on April 29, 2003, the subject matter of which is  
5 incorporated herein by reference.

### FIELD OF THE INVENTION

The present invention relates to the detection and the identification of the bacteria, *Salmonella*. In particular, the present invention relates to nucleic acid sequences for oligonucleotide primers and hybridization probes that may be used  
10 in the detection and identification of *Salmonella* by means of a polymerase chain reaction (PCR) assay or other methods of nucleic acid amplification.

### BACKGROUND OF THE INVENTION

The incidence of salmonellosis has increased significantly during the last two decades in several western countries. In general, the human population is  
15 infected by *Salmonella* via contaminated food and water, but transmission occurs, to a minor extent, by direct contact with infected animals. Standard culture methods are still widely used for the detection of *Salmonella* in foods, but control of the infection depends increasingly on the availability of rapid and precise diagnostic tests for monitoring of the primary animal production, different food  
20 processing steps, and of the final food products.

*Salmonella* can be identified from stool using cultures. Culture for *Salmonella* strains from stool, however, is time-consuming and lacks sensitivity. For the identification of *Salmonella* isolates from a stool culture, primary screening agar plates are used, sometimes in conjunction with enrichment broths. Suspect isolates, which are often present are screened using additional screening agars (e.g., triple sugar iron agar, lysine iron agar, and urea agar). Biochemical identification is then performed, and, if positive, serologic confirmation is used. Because only approximately 2% of cultured stools contain *Salmonella*, a substantial amount of work occurs due to bacterial "look-alikes" (e.g., lactose negative members of the *Enterobacteriaceae*).

*Salmonella* strains can also be identified from positive blood cultures by routine biochemical methods. In developing countries, including countries where U.S. troops may be involved, typhoid and paratyphoid fever (enteric fever) is endemic. The rapid identification of *Salmonella* directly from the blood specimen may save two days over current technology, and help distinguish enteric fever from other tropical febrile illnesses, such as malaria.

One method that can be used for the rapid identification of *Salmonella* involves a polymerase chain reaction (PCR) assay. WO 93/04202 describes a PCR assay in which polynucleotide hybridization probes and primers are targeted to the *invA*, *invB*, *invC*, and *invD* genes of *Salmonella typhimurium* for the detection of *Salmonella*. Other PCR assays for *Salmonella* target the 16S ribosomal subunit gene complex or genes that encode flagellar antigens.

### **SUMMARY OF THE INVENTION**

The present invention relates to the use of at least a portion of nucleic acid that comprises *prg* gene of *Salmonella* for the detection of all *Salmonella* strains and differentiation of these strains from other bacteria. It has been found that at least a portion of the nucleic acid sequence that comprise the *prg* genes of *Salmonella* can be used as targets for nucleic acids, which are used for the detection of all *Salmonella* strains or species. These nucleic acids are specific for *Salmonella* and do not react with other closely related enteric bacteria. Thus, these nucleic acids can serve as important clinical diagnostic agents.

Accordingly, one aspect of the present invention relates to a nucleic acid that is capable of selectively hybridizing to at least a portion of the *prg* gene. The nucleic acid that is capable of selectively hybridizing to at least a portion of the *prg* gene can be derived from the *prg* gene. The nucleic acid that is capable of selectively hybridizing to at least a portion of the *prg* gene can be used, for example, as an oligonucleotide primer or probe in a real-time PCR assay that detects at least a portion of the *prg* gene. The oligonucleotide primers and probes of the present invention can be used to differentiate *Salmonella* from other bacteria.

The present invention also relates to a method of detecting the presence of *Salmonella* in a sample. In the method, a sample suspected of including *Salmonella* is provided. A *Salmonella* target nucleotide sequence that comprises contiguous nucleotides from the *prg* gene of *Salmonella* is amplified. The amplified target nucleic acid is detected with an oligonucleotide hybridization probe, which is capable of hybridizing to the amplified target nucleotide sequence.

A further aspect of the present invention relates to a kit for use in detecting *Salmonella*. The kit comprises at least one oligonucleotide primer that includes a nucleic acid sequence that specifically hybridizes to the *prg* gene of *Salmonella*. The kit can further include a nucleic acid hybridization probe that includes a nucleic acid that specifically hybridizes to the *prg* gene of *Salmonella*.

The assays, methods, and kits of the present invention can be used to confirm the identity of isolates suspected to represent *Salmonella*. The assay, methods, and kits can rapidly identify the stool specimens that contain *Salmonella* (e.g., approximately 2% of stools submitted for culture), and more importantly can rapidly identify stool specimens that do not contain *Salmonella*, which would save labor and materials. The assays, methods, and kits can also be used by the food and veterinarian industries for the rapid identification of *Salmonella* in food products and animals, respectively. The identification of *Salmonella* in blood or blood culture in an area for endemic for enteric fever would be very helpful since enteric fever is a systemic illness with high mortality. This is in contrast to enteritis (a diarrheal disease), which is the most common form of salmonellosis in the United States. The identification of *Salmonella* by PCR can be used to screen

travelers or immigrants for colonization by *S. typhi*, or by the food or water industry, especially in developing countries where *S. typhi* is endemic.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

5 The foregoing and other features of the present invention will become apparent to one skilled in the art to which the present invention relates upon reading the following description with reference to the accompanying drawings.

FIG. 1 is a graph illustrating all *Salmonella* isolates that are detected by the Pan-*Salmonella* assay.

10 FIG. 2 is a graph illustrating that melting point analysis differentiates the *Salmonella* into three groups: a *S. typhi* containing group (left), *S. typhimurium* (right), and all other *Salmonella* (center).

FIG. 3 is a graph illustrating that the *S. typhi* specific PCR only detects *S. typhi*.

### **DETAILED DESCRIPTION**

15 The present invention may be understood more readily by reference to the following detailed description of the embodiments of the invention, and to the Examples and sequence listings included herein.

As used herein in the specification and the claims, the following terms have the given meaning unless expressly stated to the contrary.

20 "Salmonella" refers to any bacterium either currently classified or later identified in the genus *Salmonella*. *Salmonellae* are motile rods that characteristically ferment glucose and mannose without producing gas but do not ferment lactose or sucrose. The group includes three primary strains, *S. typhi*, *S. choleraesuis*, and *S. enteritidis*, and hundreds of serovars that infect a variety of  
25 different hosts. Some serotypes are primarily infective for humans, however the vast majority of salmonellae are pathogenic in animals that can serve as a source for human infection, e.g., poultry, pigs, rodents, cattle, and pets.

A "nucleotide" is a subunit of a nucleic acid consisting of a phosphate group, a 5-carbon sugar and a nitrogenous base. In DNA, the 5-carbon sugar is

deoxyribose. For a 5'-oligonucleotide, the sugar contains a hydroxyl group (-OH) at the 5' carbon.

5 The phrase "specific to", "specific for", and "unique to" the bacteria, *Salmonella*, *S. typhi*, or *S. typhimurium* as used herein in relation to a nucleic acid or nucleic acid fragment means a nucleic acid or nucleic acid fragment that is not common to other related bacteria or other microorganisms (*i.e.*, it is only present in the bacteria *Salmonella*).

10 The phrase "sample" as used herein means any sample of fluid, or of solubized or nonsolubized tissue obtained from a subject, or solubized or nonsolubized cultured cells, which contains components, such as nucleic acids or fragments thereof, that may be employed in one of the tests described herein to detect a previous or current infection by, or exposure to, the bacteria *Salmonella*, or to make a positive diagnosis of salmonellosis, enteric fever, and/or enteritis. Such samples include blood, serum, plasma, sputum, urine, mucus, saliva, gastric  
15 juice, lymph, feces, or other bodily fluids, and tissues from the lungs, spleen, liver, skin, or other organs. The samples can also be supernatant from incubated tissue sample or cultured cells.

20 The term "fragment" as used herein in relation to a nucleic acid means a sub-sequence of a nucleic acid that is of a sufficient size and confirmation to properly function as a hybridization probe or as a primer in a polymerase chain reaction (PCR) or in another manner characteristic of nucleic acids.

25 The term "hybridization" as used herein refers to the formation of a duplex structure by two single-stranded nucleic acids due to fully (100%) or less than fully (less than 100%) complementary base pairing. Hybridization can occur between fully and complementary nucleic acid strands, or between less than fully  
complementary nucleic acid strands which contain regions of mismatch due to one or more nucleotide substitutions, deletions, or additions.

30 The term "isolated" means that the nucleic acids or nucleic acid fragments are of sufficient purity so that they may be employed, and will function properly, in a clinical diagnostic, experimental or other procedure, such as a hybridization assay or an amplification reaction for *Salmonella*. Many procedures are known by those of ordinary skill in the art for purifying nucleic acids, nucleic acid fragments,

and materials with which they may normally be associated prior to their use in various procedures.

The term "substantially similar" in relation to the nucleic acid sequences of the present invention, or to the nucleotide sequences complementary the nucleotide sequences of the present invention, refers to a nucleic acid which is similar to the to the nucleic acid sequences of the present invention, or to nucleic acid sequences complementary to the nucleic acid sequences of the present invention, and which retains the functions of such nucleic acid, but which differs from such nucleic acid by the substitution, deletion, and/or addition of one or more nucleotides, and/or by the incorporation of some other advantageous feature. Nucleotide sequences of the present invention are substantially similar to a nucleic acid sequence if these percentages are from 100% to 80% or from 0 base mismatches in a 10 nucleotide sequence to 2 bases mismatched in a 10 nucleotide sequence. In some embodiments, the percentage is from 100% to 85%. In other embodiments, this percentage is from 90% to 100%; in still other embodiments, this percentage is from 95% to 100%.

The phrase "target nucleotide sequence" refers to a region of a nucleotide, which is to be amplified, detected, or otherwise analyzed. The sequence to which the oligonucleotide probe hybridizes is referred to as a target nucleotide sequence.

The present invention relates to the use of at least a portion of nucleic acid that comprises the *prg* gene of *Salmonella* for the detection of all *Salmonella* strains and differentiation of these strains from other bacteria. The *prg* gene of *Salmonella* refers to the group of *prg* genes found in *Salmonella* (e.g., *S. typhimurium* and other *Salmonella* strains), which is at least partially contributes to *Salmonella*'s cellular invasion abilities. This group of *prg* genes can include the molecular genetic complex that comprises the genes *prgH*, *prgI*, *prgJ*, *prgK*, and *orgA* gene. This genetic complex can be identified by the GenBank accession No. U21676 (SEQ ID NO: 1), which is a component of the nucleotide sequence for GenBank accession No. AE008831 (*Salmonella typhimurian* LT2, section 135 or of 220 of the complete genome; SEQ ID NO: 2) and other GenBank accessions for *Salmonella*.



It has been found that at least a portion of the nucleic acid sequence that comprises the *prg* genes of *Salmonella* can be used as a target for nucleic acids utilized in detection and differentiation of *Salmonella* (e.g., nucleic acid probes or primers). By way of example, a portion of the *prg* gene that can be used as a target in accordance with the present invention for the detection of *Salmonella* and the differentiation of *Salmonella* from other bacteria is the *prgK* gene. The *prgK* gene is believed to encode a lipoprotein that links inner and outer proteins of the *prg* gene complex. It is also believed that genetic mutations in a transmembrane protein or transmembrane region of a protein would be less tolerated than in an extracellular or cytoplasmic domain. Hence, genetic mutations in the *prgK* gene between *Salmonella* strains would also be less tolerated.

One example of at least a portion of a nucleic acid of the *prgK* gene that can be used as a target is SEQ ID NO: 3, which is located from basepair 4179 to basepair 4372 of SEQ ID NO: 2. Another example of at least a portion of a nucleic acid of the *prgK* gene that can be used as a target is SEQ ID NO: 4, which is located from basepair 165010 to basepair 165768 of GenBank accession No. AL627276, SEQ ID NO: 5. GenBank accession No. AL627276 discloses the complete genome of *S. typhi*. It will be appreciated that other regions in the *prg* gene complex can also be used as a target for the detection of *Salmonella* and the differentiation of *Salmonella* from other bacteria.

Nucleic acids that can target at least portion the *prg* gene in accordance with the present invention are capable of selectively hybridizing to at least a portion of the *prg* gene complex (e.g., *prgK* gene). These nucleic acids are specific for *Salmonella* and do not react with other closely related enteric bacteria. These nucleic acids can be derived from the *prg* gene. The derived nucleic acid is not necessarily physically derived from the *prg* gene, but may be generated in any manner including, for example, chemical synthesis, DNA replication, reverse transcription, or transcription as well as generated from RNA and peptide nucleic acids (PNAs).

A nucleic acid derived from the *prg* gene that is capable of specifically (or selectively) hybridizing to the *prg* gene of *Salmonella* in accordance with the present invention can comprises at least about 10 nucleotides. Preferably, the

derived nucleic acid can comprise about 10 to about 40 nucleotides, and more preferably about 15 to about 35 nucleotides. The derived nucleic acid can be used as a primer for the transcription and/or replication of targeted *Salmonella* sequences and/or as a probe for the detection (including isolation and/or labeling) of nucleotides, which contain *Salmonella* nucleic acid sequences. The nucleic acid can be of sufficient length and complementary with the a portion of the nucleotide sequence of the *prg* gene complex of *Salmonella* to form a duplex with sufficient stability for the purpose intended. For example, if the nucleic acids are to serve as primers for the transcription and/or replication of target nucleotide sequences of a portion of the *prg* gene complex of *Salmonella*, they should contain a nucleic acid sequence of sufficient length and complementarity to the targeted *Salmonella* sequence to allow the polymerizing agent to continue replication from the primers, which are in stable duplex form with the target sequence, under polymerizing conditions.

Nucleic acids that are capable of selectively hybridizing to at least a portion of the *prg* gene can be used, for example, as a oligonucleotide primer or probe in a real-time PCR assay that detects at least a portion of the *prg* gene of *Salmonella*. It will be appreciated by one skilled in the art the oligonucleotide probes can also be used in other assays, such as a RAPD assay or other amplification assay.

The oligonucleotide primers of the present invention serve as a priming position or initiation position for the action of primer dependent DNA polymerase activity. The oligonucleotide primers include nucleic acid sequences that are specific *Salmonella* and that can be used to amplify a target nucleotide sequence. The target nucleotide sequence is defined by contiguous nucleotides of the *prg* gene complex, such as the nucleotides of the *prgK* region (e.g., SEQ ID NO 3 or SEQ ID NO: 4). The contiguous nucleotides of *prg* complex region include contiguous nucleotides to which the oligonucleotide hybridization probes of the present invention can hybridize.

The oligonucleotide primers of the present invention can comprise a pair of oligonucleotide primers that hybridize to nucleotide sequences, which flank the target nucleotide sequence, so that DNA synthesis by the action of a DNA polymerase, such as Taq polymerase, proceeds through the region between the two

primers. This is advantageous because after several rounds of hybridization and replication the amplified target nucleotide sequence produced is a segment having a defined length whose ends are defined by the sites to which the primers hybridize.

5           An example of a pair of nucleic acid sequences that can be used for the pair of oligonucleotide primers include SEQ ID NOs: 6 and 7. SEQ ID NO: 6 is a forward primer that comprises contiguous nucleic acids from basepair 4179 to basepair 4196 of SEQ ID NO: 2. SEQ ID NO: 7 is a reverse primer that comprises contiguous nucleic acids from basepair 4372 to basepair 4355 of SEQ ID NO: 2.

10           It will be appreciated by one skilled in the art that other oligonucleotide primers of the present invention can include nucleic acid sequences complementary to SEQ ID NOs: 6-7, nucleic acid sequence substantially similar to SEQ ID NOs: 6-7, nucleic acid sequences substantially similar to a nucleic acid sequence complementary to SEQ ID NOs: 6-7, a fragment of SEQ ID NOs: 6-7  
15           that specifically hybridize to the *prg* gene complex of *Salmonella*, a fragment of a nucleic acid sequence complementary to SEQ ID NOs: 6-7 that specifically hybridize to the *prg* gene complex of *Salmonella*, a fragment of a nucleic acid sequence substantially similar to SEQ ID NOs: 6-7 that specifically hybridizes to the *prg* gene complex of *Salmonella*, and a fragment of a nucleic acid sequence  
20           substantially similar to nucleic acid sequences complementary to SEQ ID NOs: 6-7 that specifically hybridizes to the *prg* gene of *Salmonella*. It will also be appreciated that the oligonucleotide primers can include other nucleic acid sequences as long as these nucleic acid sequences specifically hybridize to the *prg* gene complex of *Salmonella*.

25           The oligonucleotide hybridization probes in accordance with the present invention are used to detect the target nucleotide sequence amplified by the oligonucleotide primers of the present invention. The oligonucleotide hybridization probes include a nucleic acid sequence that is capable of hybridizing to the amplified target amplified target nucleotide sequence of *prg* gene complex  
30           of *Salmonella*. Examples oligonucleotide probes that can hybridize to the amplified target nucleotide sequence can include at least one nucleic acid sequence comprising SEQ ID NOs: 8 and 9. SEQ ID NO: 8 is a first hybridization probe

that comprises contiguous nucleic acids from basepair 4266 to basepair 4201 of SEQ ID NO: 2. SEQ ID NO: 9 is a second hybridization probe that comprises contiguous nucleic acids from basepair 4179 to basepair 4196 of SEQ ID NO: 2.

It will be appreciated by one skilled in the art that other oligonucleotide hybridization probes of the present invention can include nucleic acid sequences complementary to SEQ ID NOs: 8 and 9, a nucleic acid sequence substantially similar to SEQ ID NOs: 8 and 9, a nucleic acid sequence substantially similar to nucleic acid sequences complementary to SEQ ID NOs 8 and 9, a fragment of SEQ ID NOs: 8 and 9 that specifically hybridize to the amplified target nucleotide sequence of the *prg* gene complex of *Salmonella*, a fragment of a nucleic acid sequence complementary to SEQ ID NOs: 8 and 9 that specifically hybridize to the amplified target nucleotide sequence of the *prg* gene complex of *Salmonella*, a fragment of a nucleic acid sequence substantially similar to SEQ ID NOs: 8 and 9 that specifically hybridizes to the amplified target nucleotide sequence of the *prg* gene complex of *Salmonella*, and a fragment of a nucleic acid sequence substantially similar to a nucleic acid sequences complementary to SEQ ID NOs 8 and 9 that specifically hybridizes to the amplified target nucleotide sequence of the *prg* gene complex of *Salmonella*. It will also be appreciated that the oligonucleotide hybridization probes can include other nucleic acid sequences as long as these nucleic acid sequences specifically hybridize to the *prg* gene complex of *Salmonella*.

The oligonucleotide hybridization probes of the present invention are preferably labeled with a detectable moiety, which can be used to detect or confirm hybridization of the oligonucleotide hybridization probes to their target sequence. The detectable moiety can be a molecule that is attached to, or synthesized as part of the oligonucleotide hybridization probe. The molecule should be uniquely detectable and allow the oligonucleotide hybridization probes to be detected as a result. Examples of detectable moieties include isotopic labels, radioactive labels, biotin, enzymes, digoxigenin, chemiluminescent labels, and fluorescent labels. The detection method selected will depend upon the hybridization conditions and detectable moiety used for labeling.

In a preferred embodiment of the present invention, fluorescence resonance energy transfer (FRET) is used to detect the oligonucleotide hybridization probes. For this detection format, two oligonucleotide hybridization probes are used that are capable of hybridizing in head-to-tail arrangement to adjacent but non-  
5 overlapping regions of the amplified target nucleotide acid sequence of the *prg* gene complex of *Salmonella*. The two oligonucleotide hybridization probes are each labeled with a respective member of fluorescent resonance energy transfer pair. One oligonucleotide hybridization probe is labeled at the 3'-end with a donor fluorophore, and the other oligonucleotide hybridization probe is labeled at the 5'-  
10 end with an acceptor fluorophore.

Fluorophore pairs that can be used as fluorescence resonance energy transfer pairs are well known to those skilled in the art. A preferred donor fluorophore is fluorescein (5-FITC), which is commercially available from SyntheGen, LLC of Houston, TX. The 3'-end of the one oligonucleotide probe can  
15 be labeled with fluorescein (5-FITC) by using a dye-derived, controlled pore glass or by post-labeling the 3'-amino modified oligonucleotide. An example of preferred acceptor fluorophore is LightCycler-Red 640 NHS ester, which is commercially available from SyntheGen, LLC. The 5'-end of the other oligonucleotide probe can be labeled with LightCycler-Red 640 NHS ester by  
20 reaction of the LightCycler-Red 640 NHS ester with a 5'-amino-modified oligonucleotide in a sodium borate buffered solution. Examples of other commercially available donor/acceptor fluorophore pairs include fluorescein/LightCycler Red 705, fluorescein/Cy7, fluorescein/Cy5, and fluorescein/Cy5.5, all of which are commercially available from SyntheGen, LLC  
25 of Houston, Texas.

If the amplified target nucleotide sequence is present, the fluorescently labeled oligonucleotide hybridization probes hybridize to the amplified target nucleotide sequence resulting in the donor and the acceptor fluorophores being separated by a distance of about 0-5 nucleotides, or more preferably 0-2  
30 nucleotides.

Fluorescent resonance energy transfer (FRET) occurs between the donor fluorophore and acceptor fluorophore when they are in physical proximity to one

another so that the donor fluorophore can transfer resonance energy to the acceptor fluorophore and the acceptor fluorophore can produce a measurable fluorescence emission. As a consequence, the hybridization can be monitored through excitation of the donor fluorophore and subsequent measurement of the fluorescence emission of the acceptor fluorophore.

When both the fluorescently labeled oligonucleotide probes are not hybridized to their complementary sequence on the amplified target nucleotide sequence, then the distance between the donor fluorophore and the acceptor fluorophore is too great for resonance energy transfer to occur. Thus, the acceptor fluorophore and the donor fluorophore are not in resonance energy transfer relationship and excitation of the donor fluorophore will not produce a detectable fluorescent emission by the acceptor fluorophore.

Examples of nucleotide sequences that can be used for the two fluorescently labeled nucleotide probes includes respectively SEQ ID NOs: 8 and 9. Pairs of nucleotide sequences that are complementary and/or substantially similar to SEQ ID Nos: 8 and 9 are also preferred.

The nucleic acids of the inventive oligonucleotide primers and hybridization probes may be made by methods well known in the art, such as chemical synthesis. The inventive oligonucleotide primers and hybridization probes may be synthesized manually or by machine. They may also be synthesized by recombinant methods using products incorporating viral and bacterial promoters.

In accordance with a method of the present invention, the oligonucleotide primers and hybridization probes can be used in a polymerase chain reaction (PCR) assay to detect and identify *Salmonella*. In the method, a sample suspected of harboring *Salmonella* is obtained. The sample may be concentrated and subjected to a procedure that partially purifies the nucleic acids in the sample.

The sample suspected of harboring *Salmonella* is then subjected to polymerase chain reaction (PCR) amplification. In PCR amplification, at least a portion of the sample is contacted with oligonucleotide primers. The oligonucleotide primers include nucleic acid sequences that are specific to *Salmonella* and that can be used to amplify a target nucleotide sequence. The

target nucleotide sequence is defined by contiguous nucleotides from the *prg* gene complex region of *Salmonella*. Preferably, the oligonucleotide primers include a pair of nucleic acid sequences that flank the target nucleotide sequence of *Salmonella* which is to be amplified. One preferred pair of nucleic acid sequences includes SEQ ID NOs: 6 and 7. Pairs of nucleic acid sequences that are complementary and/or substantially similar to SEQ ID NOs: 6 and 7, are also preferred.

PCR amplification is then conducted on the resulting mixture using a temperature program and for a number of thermal cycles sufficient to amplify the target nucleotide sequence of *Salmonella*, if present. The PCR amplification can be carried out in any commercially available PCR thermal cycling apparatus. Preferably, the PCR amplification is performed using rapid temperature cycling techniques. Rapid temperature cycling techniques use a high surface area-to-volume sample container, such as a capillary tube, to contain the reaction amplification sample. The use of a high surface-area-to-volume sample container allows for rapid temperature response and temperature homogeneity throughout the sample. Rapid temperature cycling is contrasted to conventional temperature cycling in that 30 cycles of amplification can be completed in 15 minutes and the resulting PCR amplification products contain fewer side products. Thus, with rapid temperature cycling techniques the required times for amplification are reduced approximately ten-fold, and specificity is improved.

The amplified target nucleotide sequence, if present, is then detected using an oligonucleotide hybridization probe in accordance. The oligonucleotide hybridization probe includes a nucleic acid sequence that is capable of hybridizing to the amplified DNA of *Salmonella*. Preferably, the oligonucleotide hybridization probe includes a pair of nucleic acid sequences that are labeled with a fluorescence resonance energy transfer (FRET) pair. Preferred, pairs of nucleic acid sequences, that can be fluorescently labeled include respectively SEQ ID NOs: 8 and 9. Pairs of nucleic acid sequences that are complementary and/or substantially similar to SEQ ID NOs: 8 and 9 are also preferred.

When the detection method (*e.g.*, melting point analysis) produces a result indicating that target nucleotide sequence amplified by the oligonucleotide primers

is present, it is concluded that the original sample contains *Salmonella*.

Conversely, if no evidence of the target nucleotide sequence is detected, it is concluded that sample is free of *Salmonella*.

In another aspect of the present invention, the polymerase chain reaction (PCR) amplification step and the detection step of the method are performed essentially simultaneously. Preferably, the essentially simultaneous PCR amplification step and the detection step are performed in an apparatus that includes a rapid temperature cyclers component and a fluorescent detection component. An example of such a device is described in U.S. Patent No. 6,140,540, the disclosure of which is incorporated herein by reference. The device comprises a chamber, a heater, a fan, and a carousel. The fan is mounted in the device and in air flow communication with the chamber. The carousel is rotatably mounted in the chamber and holds a plurality of sample vessels. The sample vessels used in conjunction with the device comprise an optically transparent material. The device further comprises a light emitting source and a light detector. The light emitting source is mounted in the chamber and positioned to illuminate at least one of the sample vessels. The light detector is mounted in the chamber and positioned to measure fluorescence from at least one of the sample vessels. A preferred device that includes a rapid cyclers component and fluorescent detection component is commercially available from Roche Molecular Biochemicals, of Indianapolis, IN under the trade name LIGHTCYCLER.

In yet another aspect of the invention, the detection method can provide a melt-curve profile for the disassociation of the hybridization probes to the amplified target nucleotide sequence. This melt curve profile can be compared with reference melt curve profiles for particular *Salmonella* strains (e.g., *S. typhi*) to determine the possibility of the presence of the particular *Salmonella* strain. For example, the PCR assay can provide a melt curve profile indicating the presence of *Salmonella* in a sample, and this melt curve profile can be compared with a reference melt-curve profile for *S. typhi*. Similar melt curve profiles would raise the possibility that the *Salmonella* in the sample can potentially be *S. typhi*. The sample suspect of being *S. typhi* can then be confirmed using another assay. One assay that can be used to confirm that the sample is in fact *S. typhi* comprises a



nucleic acid that is capable of selectively hybridizing to a portion of the *vexC* gene region of *S. typhi*. The *vexC* gene encodes or is associated with the vi anitgen. For GenBank accession No. AL627283, which is defined as *Salmonella enterica* serovar Typhi (*Salmonella typhi*) strain CT18, complete chromosome,  
5 segment 19/20, the *vexC* gene includes SEQ ID NO: 10. SEQ ID NO: 10 is located from basepair 41900 to base pair 42595 of GenBank AL627283, the complete sequence of which is incorporated herein by reference.

The nucleic acid that is capable of selectively hybridizing to at least a portion of the *vexC* gene can be derived from the *vexC* gene. The nucleic acid can  
10 be used for example as a nucleotide probe or primer in a real-time PCR assay that detects at least a portion of the *vexC* gene.

An example of a pair of nucleic acid sequences that can be used as a pair of oligonucleotide primers for the detection of at least a portion of the *vexC* gene include SEQ ID NOs: 11 and 12. SEQ ID NO: 11 is a forward primer that  
15 comprises contiguous nucleic acids from basepair 42395 to basepair 42410 of SEQ GenBank Accession No. AL627283. SEQ ID NO: 12 is a reverse primer that comprises contiguous nucleic acids from basepair 42610 to basepair 42595 of GenBank Accession No. AL627283.

Examples oligonucleotide probes that can hybridize to the amplified target  
20 nucleotide sequence of the *vexC* gene can include at least one nucleic acid sequence comprising SEQ ID NOs: 13 and 14. SEQ ID NO: 13 is a first hybridization probe that comprises contiguous nucleic acids from basepair 42500 to basepair 4242521 of GenBank Accession No. AL627283. SEQ ID NO: 14 is a second hybridization probe that comprises contiguous nucleic acids from  
25 basepair 42524 to basepair 42548 of GenBank Accession No. AL627283.

It will be appreciated that other oligonucleotide primers and probes that hybridize to the *vexC* gene of *S. typhi* can be used to confirm the presence of *S. typhi* in a sample suspected of being *S. typhi*. Moreover, it will be appreciated that other assay, which are not targeted to the *vexC* gene of *S. typhi*, can be used to  
30 confirm the presence of *S. typhi* in a sample suspected of being *S. typhi*.

The present invention is further directed to a kit for identifying and detecting *Salmonella* in a biological sample by means of a polymerase chain

reaction (PCR) assay. The kit includes at least one pair of oligonucleotide primers and at least one pair of oligonucleotide hybridization probes. The pair of oligonucleotide primers includes nucleic acid sequences that are specific to *Salmonella* and that can be used to amplify a target nucleotide sequence, which is defined by contiguous nucleotides from the *prg* gene complex region of the DNA of *Salmonella*.

In one example of the present invention, the kit comprises a pair of oligonucleotide primers and a pair of oligonucleotide hybridization probes. The oligonucleotide primers include at least 10 contiguous nucleotides that are capable of selectively hybridizing to at least a portion of the *prg* gene of *Salmonella*. Preferably, the oligonucleotide primers have nucleic acid sequences comprising SEQ ID NOs: 6 and 7. The oligonucleotide hybridization probes include at least 10 contiguous nucleotides and being capable of selectively hybridizing to at least a portion of the *prg* gene of *Salmonella* amplified by the hybridization probes. Preferably, the oligonucleotide hybridization probes include nucleic acid sequences having SEQ ID NOs: 8 and 9. The oligonucleotide hybridization probes are preferably labeled respectively with a donor fluorophore and an acceptor fluorophore. More preferably, the oligonucleotide hybridization probe that includes SEQ ID No. 8 is labeled at the 3'-end of the probe with Fluorescein (5-FITC), and the oligonucleotide hybridization probe that includes SEQ ID NO. 9 is labeled at the 3'-end of the probe with LightCycler Red 640.

Optionally, the kit may also contain one or all of the reagents necessary to begin the PCR amplification reaction and fluorescent detection of the oligonucleotide probes.

## EXAMPLES

The following examples illustrate use of oligonucleotide primers and oligonucleotide hybridization probes in accordance with the present invention for the amplification and detection of *Salmonella* and for the specific detection of *S. typhi* from all other bacteria, including other *Salmonella* isolates. The examples used a LIGHTCYCLER polymerase chain reaction device, which was commercially available from Roche Molecular Biochemicals of Indianapolis,

Indiana, for hybridization and probe melting studies. The oligonucleotide primers and hybridization probes were tested against 274 bacterial isolates, of which 101 were various strains of *Salmonella*. The *Salmonella* isolates tested included 23 strains of *S. typhi*, 24 strains of *S. paratyphi*, 15 strains of *S. typhimurium*, 6 strains of *S. enteritidis*, and 5 strains of *S. choleraesuis*. Other important enteric pathogens tested included 35 strains of *Shigella*, representing all four species, 27 strains of *Yersinia enterocolitica*, 12 strains of *E. coli* O157:H7, and a single *Campylobacter jejuni* isolate. The remainder of the organisms tested consisted of a variety of bacteria that may be present in clinical specimens and isolated in the clinical microbiology laboratory.

### **Materials and Methods**

A complete list of the isolates that were tested is included in Table 2. The nucleic acid was extracted from these organism and PCR with specific hybridization detection probes was carried out in the LightCycler instrument (Roche Molecular Biochemicals, Indianapolis, IN) as described below.

### **The Pan-Salmonella Assay**

The target of amplification for the pan-*Salmonella* PCR was a portion of the *prgK* gene. The GenBank entry used was AE008831 (*S. typhimurium*). The *prgK* gene is thought to encode a lipoprotein that links inner and outer membrane proteins of this complex. The *prgK* is located from basepair 4139 to basepair 4897 (GenBank Accession No. AE008831). The portion of the *prgK* gene used for this assay is located from basepair 4179 to basepair 4372. In *S. typhi* (GenBank Accession No. AL627276), the location of the *prgK* gene is 165010 to 165768 and is distinct from the *invA* gene, which has been previously used for *Salmonella* PCR, and is located from 188400 to 190457. Other regions within the *prg* gene complex would likely also be suitable for the development of a similar assay.

### **The *S. typhi* PCR assay**

The target of amplification for the *S. typhi* PCR was a portion of the *vexC* gene. The *vexC* is located from basepair 41900 to basepair 42595 (GenBank Accession No. AL627283). The portion of the *vexC* gene used for this assay is

located from basepair 42395 to basepair 42595. Other regions the *vexC* gene as well as other *vex* genes that encode the Vi antigen can potentially be suitable for the development of a similar assay.

**Oligonucleotide primers and hybridization probes**

5           The oligonucleotide primers and hybridization probes had the following nucleotide sequences and were used to target the nucleotide sequences disclosed in Table 1.

**Pan-Salmonella Assay**

Forward Primer:

10       5'-CCTTTCTTATTGCGGGCA-3' (SEQ ID NO: 6)

Reverse Primer:

5'-GCCGATGTGGATTATGAC-3' (SEQ ID NO: 7)

Hybridization Probe 1:

5'-GGATTGTTTTGATTATTTTGTATCCGTGATG-FITC-3' (SEQ ID NO: 8)

15       Hybridization Probe 2:

5'-LCRed705-AGCAGGCTTTGGCGT-P-3' (SEQ ID NO: 9)

**Salmonella typhi PCR**

Forward Primer:

5'-ACCCCGTAGCCCAATA-3' (SEQ ID NO: 11)

20       Reverse Primer:

5'-AGGAGAGACGCATTCG-3' (SEQ ID NO: 12)

Hybridization Probe 1:

5'-GCATATCGGTATTCTGGCGGC-FITC-3' (SEQ ID NO: 13)

Hybridization Probe 2:

25       5'-LCRed640-CTGGTTCAGGCAAAACGACG-P-3' (SEQ ID NO: 14)

**Table 1**

	GenBank Number	Position	Target
Pan- <i>Salmonella</i> Forward Primer	AE008831	4179-4196	PrgK gene
Pan- <i>Salmonella</i> Reverse Primer	AE008831	4372-4355	PrgK gene
Pan- <i>Salmonella</i> Hybridization Probe 1	AE008831	4266-4201	PrgK gene
Pan- <i>Salmonella</i> Hybridization Probe 2	AE008831	4179-4196	PrgK gene
<i>S. typhi</i> Forward Primer	AL627283	42395-42410	VexC gene
<i>S. typhi</i> Reverse Primer	AL627283	42610-42595	VexC gene
<i>S. typhi</i> Hybridization Probe 1	AL627283	42500-42521	VexC gene
<i>S. typhi</i> Hybridization Probe 2	AL627283	42524-42458	VexC gene

The reaction volume of 20  $\mu$ L was a mixture 5  $\mu$ L extracted target DNA and 15  $\mu$ L of Hybridization Probe master mix (Roche). These were placed together in a LightCycler<sup>®</sup> capillary tube. The LightCycler PCR parameters were used. A suspension of buffer was used as the negative control.

The presence of amplified DNA was measured by detection of energy emitted at 640 nm. The temperature at which the hybridization probes disassociated from the target DNA probe hybridization sites was determined by melting curve analysis, as provided for by the LightCycler software. This served as an independent indicator of the specificity of hybridization.

## **Results**

The results of the pan-*Salmonella* assay are shown in Table 2.

-20-

**Table 2**

Light Cycler Results  
Bacterial PCR Test Battery vs Pan *Salmonella* and  
Styphi  
Hybridization Probes

Organism (n)	Pan Salm LC result	Styphi LC result	Organism (n)	Pan Salm LC result	Styphi LC result
Staph aureus (5)	-	-	Providencia (2)	-	-
Staph epidermidis (3)	-	-	Shigella sonnei (10)	-	-
Staph saprophyticus (2)	-	-	Shigella flexneri, Group B (17)	-	-
Micrococcus (2)	-	-	Shigella boydii, Group C (6)	-	-
Stomatococcus (2)	-	-	Shigella dysenteriae, Group A (2)	-	-
Lactobacillus (2)	-	-	Burkholderia (2)	-	-
Enterococcus (3)	-	-	Yersinia kristensenii	-	-
Viridans streptococcus (3)	-	-	Yersinia enterocolitica (27)	-	-
Strep pneumoniae (3)	-	-	Citrobacter (3)	-	-
Group A streptococcus (3)	-	-	E. coli (2)	-	-
Group B streptococcus (3)	-	-	E. coli 0157 (12)	-	-
Aerococcus (3)	-	-	Proteus (3)	-	-
Listeria (3)	-	-	Klebsiella (3)	-	-
Bacillus (3)	-	-	Enterobacter (3)	-	-
Salmonella typhimurium (15)	+	-	Pseudomonas (3)	-	-
Salmonella enteritidis (6)	+	-	Acinetobacter (3)	-	-
Salmonella typhi (23)	+	+	Haemophilus (3)	-	-
Salmonella choleraesuis (5)	+	-	Neisseria meningitidis (3)	-	-
Salmonella paratyphi (24)	+	-	Neisseria gonorrhoea (3)	-	-
Salmonella agona	+	-	Non-gonococcal Neisseria sp (3)	-	-
Salmonella oslo	+	-	Moraxella (3)	-	-
Salmonella poona	+	-	Bacteroides (3)	-	-
Salmonella heidelberg (5)	+	-	Atipia felis	-	-
Salmonella infantis (8)	+	-	Vibrio cholerae	-	-
Salmonella newport (2)	+	-	Eikenella corrodens	-	-
Salmonella alachua	+	-	Pasteurella multocida	-	-
Salmonella javiana	+	-	Campylobacter jejuni	-	-
Salmonella havana	+	-	Serratia (3)	-	-
Salmonella senftenberg	+	-	Mesorhizobium hankuii	-	-
Salmonella anatum	+	-	Rhizobium sp	-	-
Salmonella saint paul	+	-	Bartonella henselae	-	-
Salmonella berta	+	-	Bartonella quintana	-	-
Salmonella braenderup	+	-	Corynebacteria (3)	-	-
Salmonella java (2)	+	-			

Total Organisms tested

274

Table 2 shows that *Salmonella* PCR/hybridization probes detected only *Salmonella*, giving 100% sensitivity and 100% specificity. No hybridization melt curves were detected for any other bacteria tested. The pan-*Salmonella* PCR amplified and detected all clinically-relevant isolates of *Salmonella* as shown in

Fig. 1. Melting point analysis determined the temperature at which the hybridization probes disassociated or melted off the target DNA sequence. Melting point analysis, therefore, was dependent upon the nucleotides present in the DNA sequence. We found that the melting point differed between the *S. typhi* and other *Salmonella* species in the pan-*Salmonella* real-time PCR assay. Fig. 2 shows that melting point analysis differentiated *Salmonella* tested into three groups: *S. typhi* (left) *S. typhimurium* (right), and all other salmonella (center). The presence of a particular melt-curve profile would therefore raise the possibility of *S. typhi*, which could be confirmed using another assay.

Table 2 also shows that the *S. typhi* assay provided specific detection of *S. typhi* from all other bacteria, including other *Salmonella* isolates. Fig. 3 shows that no hybridization melt curves were detected for any other bacteria tested besides *S. typhi*. The *S. typhi* assay amplified and detected all clinically-relevant isolates of *S. typhi*. Melting point analysis determined the temperature at which the hybridization probes disassociated or melted off the target DNA sequence. Melting point analysis, therefore, was dependent upon the nucleotides present in the DNA sequence.

Thus, the pan-*Salmonella* assay correctly detected all isolates of *Salmonella* tested and the melting curve of all the *S. typhi* isolates was distinctive from the melt curves of other *Salmonella* species. The *S. typhi* assay was positive only for the isolates of *S. typhi*. There was no cross-reactivity with the other bacteria tested.

From the above description of the invention, those skilled in the art will perceive improvements, changes and modifications. Such improvements, changes and modifications within the skill of the art are intended to be covered by the appended claims.

Having the described the invention, I claim:

1. A nucleic acid specific for use in detecting and differentiating *Salmonella* from other bacteria, said nucleic acid comprising at least 10 contiguous nucleotides and being capable of selectively hybridizing to at least a portion of the *prg* gene of *Salmonella*.
2. The nucleic acid of claim 1 being capable of selectively hybridizing to at least one of SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 4.
3. The nucleic acid of claim 1 being derived from the *prg* gene of *Salmonella*.
4. The nucleic acid of claim 1 being derived from at least one of SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 4.
5. The nucleic acid of claim 1 comprising a nucleotide sequence selected from the group consisting of SEQ ID NOs: 6-9, a sequence complementary to SEQ ID NOs: 6-9, a sequence substantially similar to SEQ ID NOs: 6-9, a sequence substantially similar to a sequence complementary to SEQ ID NOs: 6-9, and a fragment of SEQ ID NOs: 6-9, a sequence complementary to SEQ ID NOs: 6-9, or a sequence substantially similar to SEQ ID NOs: 6-9, a sequence substantially similar to a sequence complementary to SEQ ID NO: 6-9 that specifically hybridizes to *prg* gene of *Salmonella*.
6. An oligonucleotide primer specific for use in detecting and differentiating *Salmonella* from other bacteria, said primer comprising at least 10 contiguous nucleotides and being capable of selectively hybridizing to at least a portion of the *prg* gene of *Salmonella*.
7. The oligonucleotide primer of claim 6 being capable of selectively hybridizing to at least one of SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 4.



8. The oligonucleotide primer of claim 6 being derived from the *prg* gene of *Salmonella*.

9. The oligonucleotide primer of claim 6 being derived from at least one of SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 4.

10. The oligonucleotide primer of claim 6 comprising a nucleotide sequence selected from the group consisting of SEQ ID NOs: 6-7, a sequence complementary to SEQ ID NOs: 6-7, a sequence substantially similar to SEQ ID NOs: 6-7, a sequence substantially similar to a sequence complementary to SEQ ID NOs: 6-7, and a fragment of SEQ ID NOs: 6-7, a sequence complementary to SEQ ID NOs: 6-7, or a sequence substantially similar to SEQ ID NOs: 6-7, a sequence substantially similar to a sequence complementary to SEQ ID NO: 6-7 that specifically hybridizes to *prg* gene of *Salmonella*.

11. The oligonucleotide primer of claim 6, wherein the oligonucleotide primer comprises a pair of nucleic acid sequences which flank a target nucleotide sequence of *Salmonella*.

12. The oligonucleotide primer of claim 3, wherein the pair of nucleic acid sequences includes SEQ ID NO: 6 and SEQ ID NO: 7.

13. An oligonucleotide hybridization probe specific for use in detecting and differentiating *Salmonella* from other bacteria, said probe comprising at least 10 contiguous nucleotides and being capable of selectively hybridizing to at least a portion of the *prg* gene of *Salmonella*.

14. The oligonucleotide hybridization probe of claim 13 being capable of selectively hybridizing to at least one of SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 4.

15. The oligonucleotide hybridization probe of claim 13 being derived from the *prg* gene of *Salmonella*.
16. The oligonucleotide hybridization probe of claim 13 being derived from at least one of SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 4.
17. The oligonucleotide hybridization probe of claim 13, wherein said nucleic acid sequence is labeled with a detectable moiety.
18. The oligonucleotide hybridization probe of claim 17 wherein the detectable moiety is a fluorescent label.
19. The oligonucleotide hybridization probe of claim 13 wherein the oligonucleotide hybridization probe is detectable by fluorescence resonance energy transfer.
20. The oligonucleotide hybridization probe of claim 13 comprising a nucleotide sequence selected from the group consisting of SEQ ID NOs: 8-9, a sequence complementary to SEQ ID NOs: 8-9, a sequence substantially similar to SEQ ID NOs: 8-9, a sequence substantially similar to a sequence complementary to SEQ ID NOs: 8-9, and a fragment of SEQ ID NOs: 8-9, a sequence complementary to SEQ ID NOs: 8-9, or a sequence substantially similar to SEQ ID NOs: 8-9, a sequence substantially similar to a sequence complementary to SEQ ID NO: 8-9 that specifically hybridizes to *prg* gene of *Salmonella*.
21. The oligonucleotide primer of claim 13, wherein the oligonucleotide primer comprises a pair of nucleic acid sequences which flank a target nucleotide sequence of *Salmonella*.
22. The oligonucleotide hybridization probe of claim 21, wherein the pair of nucleic acid sequences includes SEQ ID NO: 8 and SEQ ID NO: 9.

23. A method of detecting the presence of *Salmonella* in a sample, said method comprising the steps of:

providing a sample suspected of including *Salmonella*;

amplifying a target nucleotide sequence using an oligonucleotide primer, the target nucleotide sequence comprising at least a portion of the *prg* gene of *Salmonella*; and

contacting the amplified target nucleic acid with an oligonucleotide hybridization probe which is capable of hybridizing to the amplified target nucleotide sequence.

24. The method of claim 23 wherein said amplifying step is performed using polymerase chain reaction in a rapid temperature cycler.

25. The method of claim 23 wherein the amplified target nucleotide sequence is detected by fluorescence.

26. The method of claim 23 wherein the amplified target nucleotide sequence is detected by at least one fluorescently labeled oligonucleotide hybridization probe.

27. The method of claim 23 wherein the amplified target nucleotide sequence is detected by two oligonucleotide hybridization probes, each labeled with a fluorescent moiety, such that when both probes are hybridized to the target nucleotide sequence, fluorescence resonance energy transfer occurs between the fluorescent moieties.

28. The method of claim 27 wherein the oligonucleotide hybridization probe comprises a nucleic acid sequence that is derived from at least one of SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 4.

29. The method of claim 27 wherein the pair of oligonucleotide hybridization probes comprises, respectively, SEQ ID NO: 8 and SEQ ID NO: 9.

30. A kit for use in detecting *Salmonella*, said kit comprising at least one of:

a primer comprising at least 10 contiguous nucleotides and being capable of selectively hybridizing to at least a portion of the *prg* gene of *Salmonella*; and

a nucleic acid hybridization probe comprising at least 10 contiguous nucleotides and being capable of selectively hybridizing to at least a portion of the *prg* gene of *Salmonella*.